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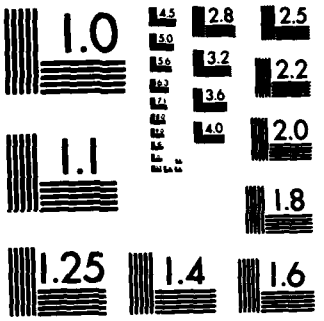

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NR 307-348

FINAL TECHNICAL REPORT TO THE OFFICE OF NAVAL RESEARCH

ONR CONTRACT N00014-80-C-0029

"FORMATION AND CHARACTERIZATION OF BACTERIAL MAGNETITE"

University of New Hampshire
Durham, N.H. 03824

Dr. Richard P. Blakemore. Principal Investigator

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CONTENTS

A. OBJECTIVES

The objectives cited in the research proposal for the ONR contract N00014-80-C-0029 included:

1. Optimization of bacterial magnetite production using pure cultures of Aquaspirillium magnetotacticum.
2. Development and evaluation of a laser technique for directly measuring cell magnetic moments from magnetic field-dependent culture birefringence.
3. Continued studies directed at elucidating the biochemical pathway of bacterial magnetite formation.
4. Analysis of the structure and function of the magnetosome sheath (e.g. the envelope surrounding each magnetite grain).
5. Initiation of studies of metal solubilization and uptake by magnetotactic bacteria.

Sufficient funds were awarded to cover the cost of part of these objectives although the period of the contract was shortened from the three years requested, to two. The latter two objectives were eliminated from the revised work plan. One (# 4) has been adopted as a research goal of BioMagnetech Corporation, as part of a proposal presently under review by the Defense Advanced Research Projects Agency.

Several reports have been submitted describing progress of ONR sponsored research carried out under this contract. These include Technical Report No. 1 submitted July 1980 (see Appendix I), and a Progress Report Abstract of Oct. 28, 1981 (see Appendix II). This report details the results of research activities not covered in either of these preceding reports, and serves as the Final Technical Report for the contract and its Field Modifications.

B. COMPLETED RESEARCH ACTIVITIES

1. Optimization of bacterial magnetite yield: Results of early studies indicated that cells of each type of magnetotactic bacterium found in natural populations produced a more-or-less constant quantity of magnetite. Remagnetization curves (plots of % cells remagnetized as a function of the remagnetizing field strength) obtained by exposing cell populations to brief. monophasic magnetic pulses, were sharp and showed a narrow distribution about the mean. Cell populations of a given type appeared homogeneous with respect to their magnetic moment distribution (12), and when examined by electron microscopy. cells appeared to have a rather consistent number of magnetosomes (12). Why should this be so? The most obvious explanations are that cells somehow regulate their production of magnetite in response to environmental parameters, or that the number of magnetosomes per cell is genetically controlled.

We addressed this question using pure cultures of the magnetic spirillum A. magnetotacticum (1) with the overall goal of optimizing magnetite yield by cells in controlled culture situations. We learned, not surprisingly, that cell magnetite yield was influenced by culture iron supply. With no added iron in the culture medium. cells each produced zero to several magnetosomes (Fig 1A) and the magnetosome content increased in direct proportion to the iron supply as shown in Fig. 2. By increasing the iron supply to 20 uM, cultures were obtained with an average of 8 magnetosomes per cell (Fig. 2), while at 40 uM iron they produced an average of 40 magnetosomes per cell (Fig 1B). Thus, the absolute number of magnetosomes produced per cell

did not appear to be genetically controlled. Even without genetic manipulation, cells may eventually be produced with extremely high numbers of magnetosomes if iron precipitation in the medium at high concentration can be avoided using chelators that keep it solubilized but do not also limit its availability to the bacteria.

It soon became apparent, that O_2 influences cell magnetite production (4;12;17). Thus, at high O_2 cells eventually grew but did not produce magnetosomes and were not magnetotactic. At a constant, non-limiting iron supply, cells in one study produced an average of 18 magnetosomes (Balkwill, Maratea and Blakemore - referenced in 4) whereas in another carried out at the same iron concentration but at lower Po_2 (unpublished data shown in Fig. 2), they produced an average of only 8 magnetosomes per cell.

We have recently begun an extensive and comprehensive study of the effect of O_2 on cell magnetite formation and yield. The results to date (12.17) have not only confirmed the repressive effect of high Po_2, but have also revealed that cells will not produce magnetite when the initial Po_2 is below about 1 kPa (1% of saturation). These unexpected results are probably going to be of rather broad interest. Geologists have lamented the lack of fossils which are indicative of free atmospheric O_2. Thus, the study of the fine structure of the evolution of earth's early atmosphere during the Archaen-Early Proterozoic era would be advanced if such fossil evidence existed. Kirschvink and Chang (cited in 12) reported bacterial magnetosomes as microfossils preserved in sediments. If this is true then bacterially derived sediment/rock magnetite could become useful fossil and

paleomagnetic evidence for deposits formed only after free O_2 became available on earth - possibly when the earth's atmosphere was, by today's standards, microaerobic. This is also interesting in the light of our growing conviction that all magnetotactic bacteria are fastideous microaerophiles.

A second finding from this study of O_2 effects pertained to culture nitrogen supply. Cells produced magnetite optimally when they were denitrifying (e.g. using NO_3^- as a terminal electron acceptor) in contrast to respiring with O_2 as the terminal acceptor (12;17). This suggested that culture magnetite yields were related to the principal electron acceptor used by cells and to their mode of electron transport.

Our investigations of the manner in which cells reduce nitrate started out as peripheral to the goals of ONR sponsored research but took on a more central role as it became apparent that cell nitrogen supply affected cell magnetosome yield. This work (D.A.Bazylinski, 1983. Ph.D. thesis U.N.H.; 6;13;14) revealed that cells denitrify with the production of nitrous oxide (N_2O) and dinitrogen (N_2). Nitrite. which was toxic for cells, did not accumulate; presumably because the nitrate reductase activity was lower than the rate of nitrite and nitrous oxide reduction. Of particular interest, we found that cells of the microaerophile A. magnetotacticum denitrify in the presence of low amounts of oxygen. This organism was one of only three which have been shown to do so - all within the past year. Our studies. however. are the first which document that a single organism with an obligate requirement for O_2 can concomitantly denitrify and respire aerobically. Thus, the regulation of this

microaerophile's nitrogen reducing enzymes by O_2 appears to differ from that of other denitrifyers, and the findings also emphasize that this organism is an obligate microaerophile and will not grow anaerobically even with nitrate in its medium.

We looked for. and found, iron reductase activity in magnetic bacteria (16). As yet we have not determined the role of iron reduction by these cells although we are interested in determining whether nitrate reductase of the magnetc spirillum will function in the reduction of iron - perhaps in a process related to magnetite formation. This might explain why denitrifying cells are especially adept at producing magnetite. for instance. Although preliminary results (16) have suggested that the nitrate and iron reductase activities were separate in the magnetic spirillum, we are continuing research in this area at the present time. Evidence for iron reduction was also obtained from analysis of cells by Mossbauer spectroscopy (5;8;9).

Results of a related study addressing cell nitrogen metabolism indicated that cells of A. magnetotacticum were nitrogen fixers (7). Moreover. their nitrogen fixation rates were found to be greater than those of Azospirillum lipoferum, the organism now receiving considerable attention in biotechnological circles as possibly being useful in agriculture.

Our explorations of cell iron and nitrogen metabolism have opened up new areas of research, some of which we hope to pursue if a research contract proposal entitled "Iron Associated Outer Membrane Proteins of Magnetic Bacteria" now before ONR, proves of value to Naval objectives.

2. **Development of an optical method for directly measuring cell magnetic moments:** Working collaboratively with Dr. Richard Frankel and Dr. Charles Rosenblatt of the Francis Bitter National Magnet Laboratory of M.I.T., we have developed, tested and used a new method of determining cell magnetism. This technique is based upon directly measuring the magnetic field-dependence of the optical birefringence of magnetic cells suspended in their culture medium. This proved to be a successful technique and it was applied in the study of the effect of oxygen upon cell magnetism referred to above. Although some effort to duplicate the necessary laser instrumentation using donated outdated or borrowed eqipment was undertaken here at UNH, this was later abandoned in favor of preserving cells in fixative and shipping them to M.I.T. for analysis. Recently we have been using electron microscopy to evaluate cell magnetosome yield in conjunction with these measurements. The optical birefringence methods do not give information concerning the proportion of cells in the population which are not at all magnetic; they only provide information concerning the relative magnetism of the magnetic cell fraction. In lieu of fabricating a second setup, we have recently decided that to obtain information concerning culture magnetism we will place more emphasis upon the use of chemically fixed culture samples for electron microscopy using negative staining.

3. **The pathway of bacterial magnetite formation:** The second major activity carried out under the contract was an investigation of the manner in which cells produce magnetite from soluble (chelated) iron supplied in their culture medium. The bacterial

system has provided a splendid opportunity to examine biogenic magnetite formation and the results have been most rewarding. This work (5;8) has been carried out collaboratively with Dr. Richard Frankel of M.I.T. We prepared cells enriched in ^{57}Fe by growing them under controlled aeration using a galvanic oxygen electrode. The cells were harvested and fractionated according to a prearranged protocol and the various cell fractions were analyzed by means of Mossbauer resonance spectroscopy in Dr. Frankel's laboratory. Wild type magnetic cells were analyzed as were wild type cells harvested early (before they had become magnetic). In addition, we obtained and analyzed mutants which were not magnetotactic and which did not produce magnetite under any conditions. The mutants were obtained by growing cells in very strong, permanent inhomogeneous magnetic fields. A screening method was developed making use of the magnetotactic response of cells containing magnetite. (see Fig.3). Methods for culturing fastideous microaerophiles on plates of semisolid media had to be developed. All work had to be conducted in a microaerobic atmosphere maintained within a special glovebox equipped with an oxygen electrode used to continuously monitor the Po_2.

The results of this joint research have provided us with the first overall understanding of how bacteria produce magnetite at temperatures and pressures compatible with life (5;8;9;). Cells appear to first take up ferric iron from chelates in their environment and reductively release it in the cell interior. Ferrous iron is apparently rapidly reoxidized and deposited intracellularly as a low density hydrous ferric oxide. This

oxidation step especially if mediated by a dioxygenase enzyme. may be the point at which molecular oxygen is required for magnetite formation. The low density hydrous iron oxide is apparently converted via dehydration to a high density ferric oxide. This material has the characteristics of ferrihydrite. the form in which iron occurs within the cores of the iron storage compound, ferritin. The final step in bacterial magnetite formation appears to involve reduction of 1/3 of the iron atoms in ferrihydrite and further dehydration to form magnetite. The partially purified bacterial magnetosome fraction which contained the magnetosome sheaths, also contained ferrihydrite. In a more recent study of the process of magnetite crystal growth (11), evidence was obtained that an amorphous gel-like region surrounding each magnetite grain consisted of the hydrated iron (III) oxide phases identified by Mossbauer spectroscopy. These observations suggest an important role of the magnetosome sheath in the physics and chemistry of magnetite formation. The involvement of ferrihydrite/ferritin in bacterial magnetite formation is especially interesting. There are only three prokaryotes known at present to contain ferritin. Azotobacter vinlandii has a recently discovered bacterioferritin which is also a cytochrome. Escherichia coli has an iron storage protein with the characteristics of ferritin, and our studies have brought to light a ferrritin-like material in magnetic bacteria. The significance of these iron compounds or the extent of their distribution among prokaryotes is not at all clear at the present time.

The details of magnetite formation by bacteria (the manner

In which cells obtain iron and reductively move it into the cell compartment, the role of the magnetosomes sheath, the enzyme activities involved, the possible participation of electron transport to iron, the possible role of proton motive force, the chemical basis for the O_2 requirement. the cell structural features which control the intracellular location of magnetosomes, whether the chemical composition or the morphology of the bacterial magnetosome can be controlled and diverse other questions) have yet to be worked out. Nevertheless. the overall process as we now understand it appears remarkably similar to that employed by chitons (primitive marine molluscs). These are the only other organisms for which the chemistry of magnetite formation has been worked out.

Detailed methods, results and conclusions of the contract-supported work are provided in the published manuscripts and reports which have emanated from the research. These are listed as references in section D of this report entitled PUBLICATIONS. and are compiled in APPENDIX III.

C. FIGURES AND LEGENDS

FIGURE 1. Aquaspirillum magnetotacticum cultured at two iron concentrations.

FIGURE 1A.

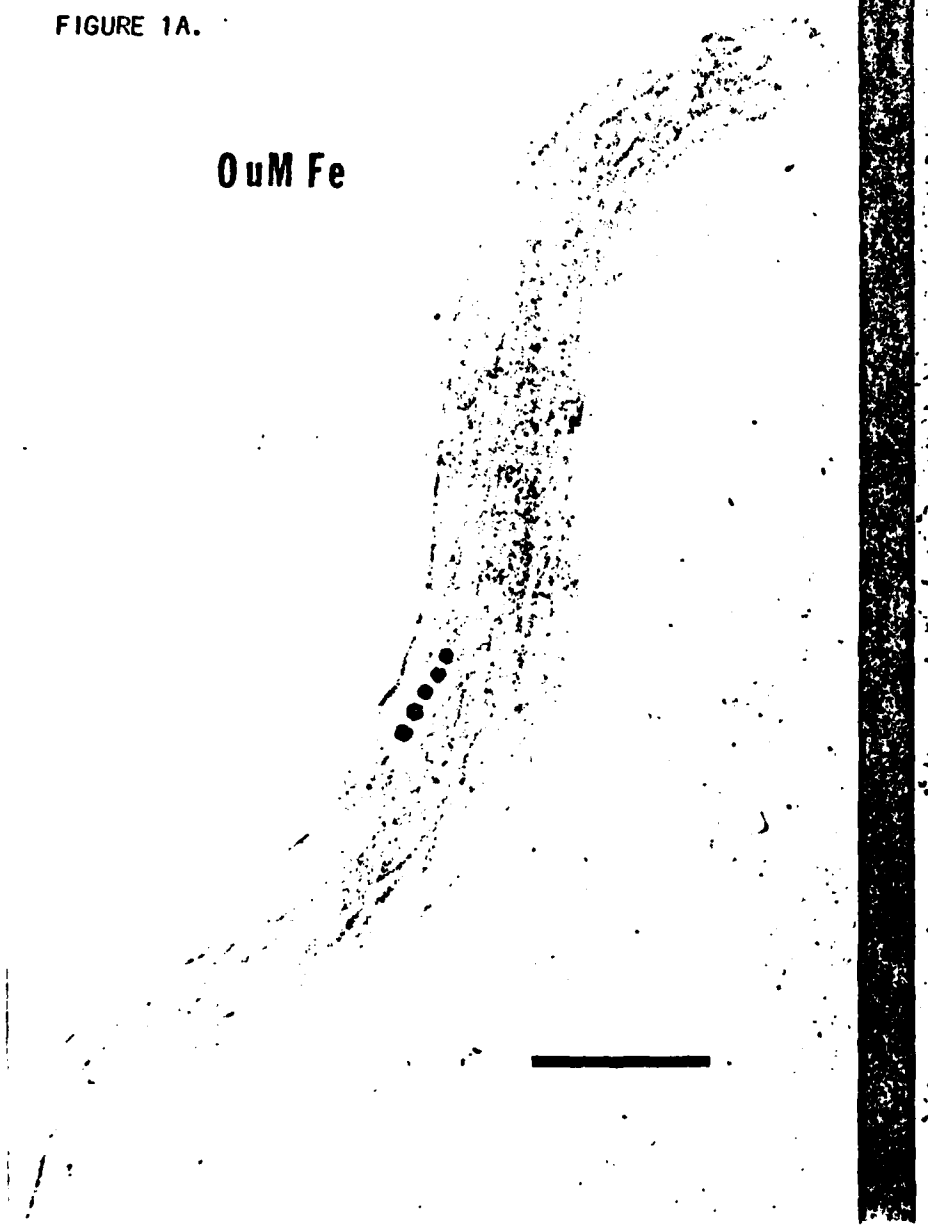
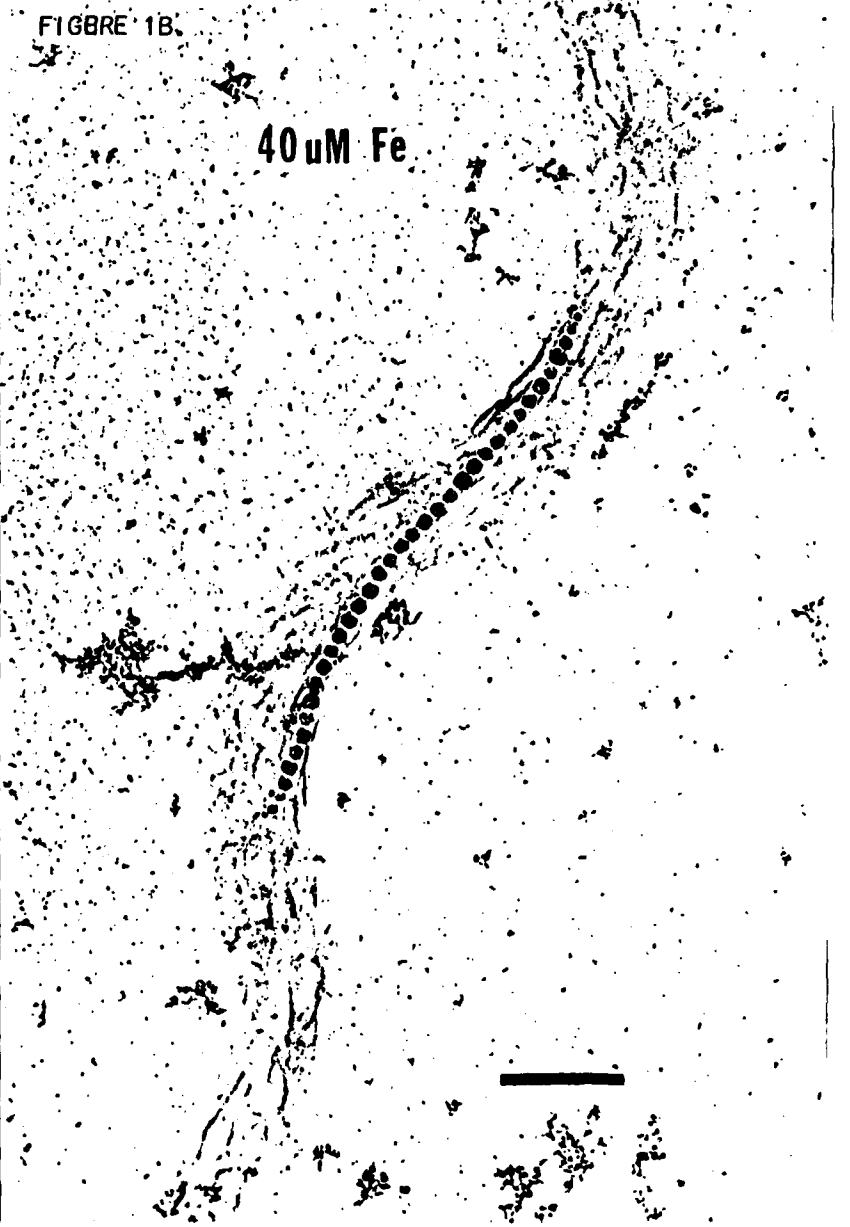


FIGURE 1B.



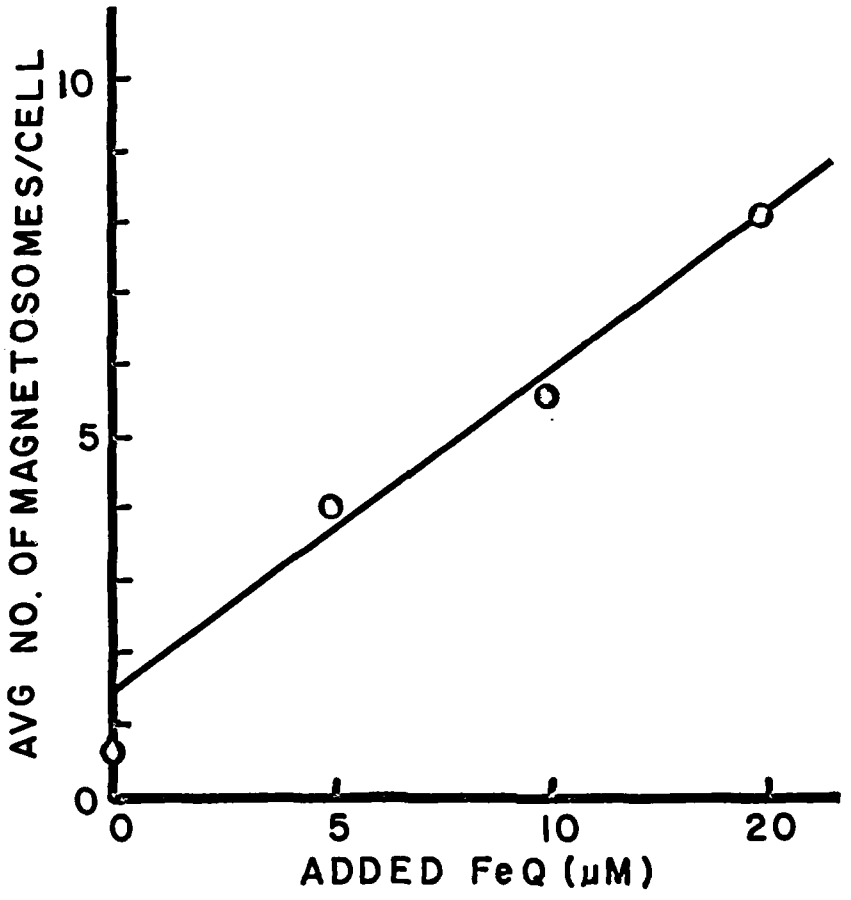


FIGURE 2. Effect of culture iron concentration on cell magnetosome production.

FIGURE 3. Screening method used to obtain non-magnetotactic bacterial mutants. Colonies containing magnetic bacteria are ellipsoid because cells run in the direction of the magnetic field applied to the Petri dish. Non-magnetic cells produce spherical colonies (arrow).

D. PUBLICATIONS

The published findings of work supported by this contract are compiled in APPENDIX III. These include:

PUBLISHED REFEREED PAPERS:

1. Maratea. D. and R.P. Blakemore. 1981. Aquaspirillum magnetotacticum sp. nov., a magnetic spirillum. Int. J. Syst. Bacteriol. 31:452-455.

2. Frankel, R.B., R.P. Blakemore. F.F. Torres de Araujo, D.M.S. Esquivel, and J. Danon. 1981. Magnetotactic bacteria at the geomagnetic equator. Science 212:1269-1270.

3. Blakemore, R.P. and R.B. Frankel. 1981. Magnetic Navigation in Bacteria. Scientific American 245:58-65.

4. Blakemore, R.P. 1982. Magnetotactic Bacteria, Ann. Rev. Microbiol. 36:217-238.

5. Frankel,R.B., G.C.Papaefthymiou, R.P. Blakemore and W. O'Brien. 1983. Fe_3O_4 Precipitation in Magnetotactic Bacteria. Biochim. Biophys. Acta 763:147-159.

6. Bazylinski,D.A. and R.P. Blakemore. 1983. Denitrification and Assimilatory Nitrate Reduction in Aquaspirillum magnetotacticum. Appl. Environ. Microbiol. 46:1118-1124.

7. Bazylinski,D.A. and R.P. Blakemore. 1983. Nitrogen Fixation (Acetylene Reduction) in Aquaspirillum magnetotacticum Current Microbiol. 9:305-308.

8. Frankel,R.B. and R.P. Blakemore. 1984. Precipitation of Fe_3O_4 in magnetotactic bacteria. Phil. Trans. R. Soc. Lond. 304:567-574.

9. Frankel, R.B., G.C.Papaefthymiou and R.P.Blakemore. 1984. Mossbauer spectroscopy of iron biomineralization products in magnetotactic bacteria. In: Magnetite biomineralization and magnetoreception by organisms: A new magnetism. J.L. Kirschvink, D.S. Jones, and B. McFadden (eds.) Plenum Press. N.Y.

10. Ofer, S., I.Nowik, E.R. Bauminger, G.C. Papaefthymiou, R.B. Frankel and R.P. Blakemore. 1984. Magnetosome dynamics in magnetotactic bacteria. Biophys. J. 46:57-64.

11. Mann, S., R.B. Frankel and R.P. Blakemore. 1984. Structure. morphology and crystal growth of bacterial magnetite. Nature 310:405-407.

12. Blakemore, R.P., K.A. Short, D.A. Bazylinski, C. Rosenblatt and R.B. Frankel. 1984. Microaerobic conditions are required for magnetite formation within Aquaspirillum magnetotacticum. Geomicrobiology J. 4:62-66.

PUBLISHED ABSTRACTS AND PROCEEDINGS:

13. Bazylinski, D.A. and R.P. Blakemore. 1982. Nitrogen metabolism in Aquaspirillum magnetotacticum. Abstr. 82nd Ann. Meet. Am. Soc. for Microbiol. 153, p. 103.

14. Bazylinski, D.A. and R.P. Blakemore. 1982. The role of magnetic bacteria in the cycling of nitrogen and iron. Abstr. New Engl. Estuarine Res. Soc.

15. Blakemore, R.P. 1982. Magnetite formation by bacteria. EOS Trans. Am. Geophys. Union 62:849-850.

16. Bazylinski, D.A. and R.P. Blakemore. 1983. Nitrate, nitrite and iron reduction in cell-free extracts of Aquaspirillum magnetotacticum. Abstr. 6th Int'l. Sympos. Environ. Biogeochem. Santa Fe, N.M.

17. Bazylinski, D.A., K. Short and R. Blakemore. 1984. Microaerobic conditions are required for magnetite formation within Aquaspirillum magnetotacticum. Abstr. 84th Annu. Mtg. Am. Soc. for Microbiol. St. Louis, MO. I 95.

18. Bazylinski, D.A., E. Palome and R.P. Blakemore. 1984. Denitrification by Chromobacterium violaceum. Abstr. 84th Annu. Mtg. Am. Soc. for Microbiol. St. Louis, MO. I 92.

19. O'Brien, W., L. Paoletti and R. Blakemore. 1984. Cytochromes in Aquaspirillum magnetotacticum. Abstr. 84th Annu. Mtg. Am. Soc. for Microbiol. St. Louis, MO. K 45

THESES CONTAINING WORK SUPPORTED BY THE CONTRACT

1. A STUDY OF IRON UPTAKE IN AQUASPIRILLUM MAGNETOTACTICUM. 1982. Mark Berman - Master of Science.

2. THE TERMINAL RESPIRATORY CHAIN COMPONENTS OF AQUASPIRILLUM MAGNETOTACTICUM. 1982. Wendy O'Brien - Master of Science.

3. NITROGEN METABOLISM AND IRON REDUCTION IN AQUASPIRILLUM MAGNETOTACTICUM. 1984. Dennis Bazylinski - Ph.D.

4. IRON ASSIMILATION IN AQUASPIRILLUM MAGNETOTACTICUM. 1984. Lawrence Paoletti - Master of Science.

APPENDICES

1. APPENDIX I - TECHNICAL REPORT NO. 1 JULY, 1980

2. APPENDIX II - PROGRESS REPORT ABSTRACT OCT., 1981

3. APPENDIX III - COMPILED PUBLICATIONS

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